US Environmental Protection Agency
Office of Pesticide Programs

Office of Pesticide Programs
Microbiology Laboratory
Environmental Science Center, Ft. Meade, MD

Standard Operating Procedure for
Neutralization of Microbicidal Activity
using the OECD Quantitative Method for
Evaluating Bactericidal Activity of Microbicides
Used on Hard, Non-Porous Surfaces

SOP Number: MB-26-00

Date Revised: 03-14-13

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Title	Neutralization of Microbicidal Activity using the OECD Quantitative Method for Evaluating Bactericidal Activity of Microbicides Used on Hard, Non-Porous Surfaces
Scope	To verify the neutralization efficacy of a test substance. This SOP is based on Annex II of OECD test guidelines dated January 29, 2013 (see ref. 15.1).
Application	Identify a suitable neutralizer in advance of or concurrently with testing. Verify neutralization using the highest concentration of test substance if there are multiple concentrations being evaluated.

	Approval Date
SOP Developer:	_____ Print Name: _____
SOP Reviewer	_____ Print Name: _____
Quality Assurance Unit	_____ Print Name: _____
Branch Chief	_____ Print Name: _____

Date SOP issued:	
Controlled copy number:	
Date SOP withdrawn:	

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1. Definitions	Additional abbreviations/definitions are provided in the text. 1. Eluent = any liquid that is harmless to the test organism(s) and that is added to a vial containing the carrier to recover the test organism. 2. Eluate = recovered eluent that contains the test organism. 3. Test suspension = suspension of the test microbe prior to the addition of the soil load (*Test Suspension A*) 4. Final test suspension= test suspension with soil load (*Test Suspension B*) 5. Stock culture = frozen culture used to prepare the test culture 6. Test substance = a product or formulation that is under evaluation for its microbicidal activity
2. Health and Safety	Follow procedures specified in SOP MB-01, Laboratory Biosafety. The Study Director and/or lead analyst should consult the Material Safety Data Sheet for specific hazards associated with products.
3. Personnel Qualifications and Training	Refer to SOP ADM-04, OPP Microbiology Laboratory Training.
4. Instrument Calibration	Refer to SOP EQ-01, EQ-02, EQ-03, EQ-04 and EQ-05 for details on method and frequency of calibration.
5. Sample Handling and Storage	Refer to SOP MB-22, Disinfectant Sample Preparation, and SOP COC-01, Chain of Custody Procedures.
6. Quality Control	For quality control purposes, the required information is documented on the appropriate form(s) (see section 14).
7. Interferences	Prolonged exposure of cells to the neutralizer agent in excess of 30 minutes may result in erroneous values due to bacterial replication; timely filtration will mitigate this potential interference.
8. Non-conforming Data	Management of non-conforming data will be specified in the study protocol; procedures will be consistent with SOP ADM-07, Non-Conformance Reports.
9. Data Management	Data will be archived consistent with SOP ADM-03, Records and Archives.
10. Cautions	Avoid extended soaking of the carriers in water or detergent and prolonged rinsing to reduce risk of corrosion or rusting.
11. Special Apparatus and	Refer to SOP MB-25, section 11.

Materials	
12. Procedure and Analysis	1. General description of the assay: a. The test substance is first mixed with a candidate neutralizer. The test organism is then added to the reaction mixture as dried inoculum on a carrier; if desired, additional evaluations may be conducted using the test organism as a liquid. The neutralization process is deemed acceptable if the criteria outlined in section 13 are met.
12.1 Preparation/ sterilization of carriers	a. Refer to SOP MB-25, section 12.1.
12.2 Preparation of test organisms	a. Refer to SOP MB-25; section 12.2a through 12.2i for *P. aeruginosa* and *S. aureus*, Attachment 2 section A4.a through A4.e for *M. terrae*. b. Prepare *Test Suspension A (without soil load)*: Dilute the test microbial suspension with PBS to achieve an average challenge of 20-200 CFU/carrier after the 10 µL inoculum has dried (e.g., serially dilute 10 mL cultures of *P. aeruginosa* through 10^{-3}, serially dilute 10 mL cultures of *S. aureus* through 10^{-4}, serially dilute 5 mL cultures of *M. terrae* through 10^{-2}; refer to the Neutralization Test Suspension Preparation Sheet in section 14.0). Prior testing may be required to account for differences in the loss of viability of the different test organisms upon drying. *Test Suspension A* should be used within 4 hours of preparation. c. Prepare *Final Test Suspension B (with soil load)*: Prepare the soil load: vortex each component and combine 25 µL bovine serum albumin (BSA), 35 µL yeast extract, and 100 µL of mucin, mix well. Combine 132 µL of *Test Suspension A* and 68 µL of the soil load (SL). The test microbial suspension with soil load should also achieve an average challenge of 20-200 CFU/carrier after drying. Note: Two separate serial dilutions of *Test Suspension A* may be used to prepare two different concentrations of *Final Test Suspension B* to ensure at least one set of carriers with an average challenge of 20-200 CFU/carrier after the inoculum has dried. Thus, the use of two separate dilutions results in a total of 20 carriers to be processed; however, the dilutions may be evaluated separately. d. It is recommended that a calibration curve, using optical density (OD @ 650nm), be created to estimate the number of viable

		organisms in *Test Suspension A*.
12.3	Carrier inoculation	a. Inoculate at least 13 carriers with *Test Suspension A* (if conducting optional treatments) and at least 13 carriers with *Final Test Suspension B* (per concentration of *Final Test Suspension B*) by adding 10 µL using a positive displacement pipette to each carrier. Refer to SOP MB-25, sections 12.4c through 12.4d for carrier drying instructions. b. After drying, evaluate the dried carriers using the required treatments (section 12.4). Optional treatments (section 12.5) may be evaluated if necessary.
12.4	Required treatments	a. ***Treatment 1a: Titer Control (with SL).*** Add 10 mL PBS to each of four vials. At timed intervals, add one dried carrier inoculated with *Final Test Suspension B* gently to each vial. Vortex each vial for 30 ± 2 s. Proceed with section 12.6. If necessary, using another vial, perform this procedure with 10 µL of *Final Test Suspension B* in place of one of the inoculated carriers. b. ***Treatment 2: Neutralizer Toxicity Control (with SL).*** Add 10 mL neutralizer to each of three vials. At timed intervals, add one dried carrier inoculated with *Final Test Suspension B* gently to each vial. Vortex each vial for 30 ± 2 s. Proceed with section 12.6. If necessary, using another vial, perform this procedure with 10 µL of *Final Test Suspension B* in place of one of the inoculated carriers. c. ***Treatment 3a: Neutralizer Effectiveness (with SL).*** Add 50 µL of the test substance to each of three vials. At timed intervals, add 10 mL neutralizer to each vial and briefly swirl. After 10 s, gently add one dried carrier inoculated with *Final Test Suspension B* to each vial. Vortex each vial for 30 ± 2 s. Proceed with section 12.6. If necessary, using another vial, perform this procedure with 10 µL of *Final Test Suspension B* in place of one of the inoculated carriers.
12.5	Optional treatments	a. ***Treatment 1b: Optional Titer Control (without SL).*** Add 10 mL PBS to each of four vials. At timed intervals, add one dried carrier inoculated with *Test Suspension A* gently to each vial. Vortex each vial for 30 ± 2 s. Proceed with section 12.6. If necessary, using another vial, perform this procedure with 10 µL of *Test Suspension A* in place of one of the inoculated carriers. b. ***Treatment 3b: Optional Neutralizer Effectiveness (without SL).*** Add 50 µL of the test substance to each of three vials. Then at timed intervals, add 10 mL neutralizer each vial and briefly swirl. After 10 s, gently add one dried carrier inoculated with *Test Suspension A*

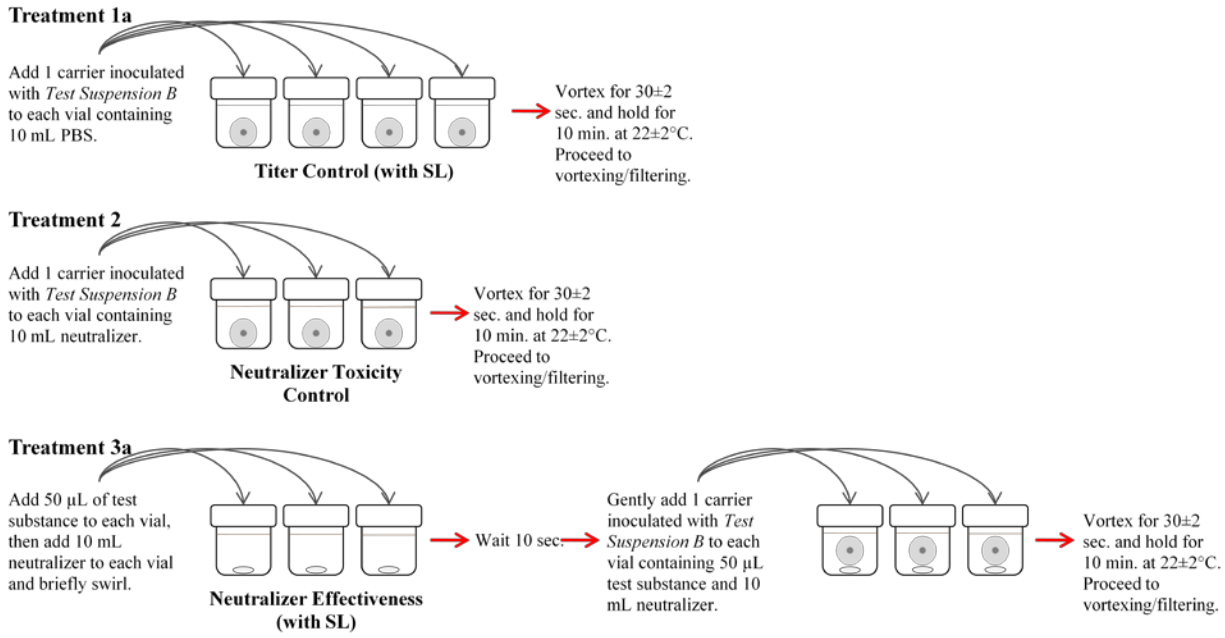
	to each vial. Vortex each vial for 30 ± 2 s. Proceed with section 12.6. If necessary, using another vial, perform this procedure with 10 µL of *Test Suspension A* in place of one of the inoculated carriers. c. ***Treatment 3c: Optional Neutralizer Effectiveness (independent addition of SL).*** Add 50 µL of the test substance to each of three vials. At timed intervals, add 10 mL of the neutralizer to each vial followed by the addition of 10 µL of the 3-part soil load to each vial and briefly swirl. After 10 s, add one dried carrier inoculated with *Test Suspension A* to each vial. Vortex each vial for 30 ± 2 s. Proceed with section 12.6. If necessary, using another vial, perform this procedure with 10 µL of *Test Suspension A* in place of one of the inoculated carriers.
12.6 Processing and recovery	a. Hold the mixtures from 12.4 and 12.5 for 10 ± 1 min at room temperature (22±2°C). Steps (e.g., addition of PBS, neutralizer) should be conducted at timed intervals (e.g., 1 min. intervals) to ensure consistent time of contact. b. At the conclusion of the holding period, vortex each vial for 30 ± 2 s and pass each mixture through a separate, pre-wetted 0.2 or 0.45 µm polyethersulfone (PES) membrane filter. Use a magnet to prevent carriers from falling onto the filter membrane. Wash each vial with approximately 20 mL PBS and vortex for 5 ± 1 s; filter the washes through the same filter membrane. Repeat once (altogether 40 mL of PBS). Finish the filtering process by rinsing the inside of the funnel unit with about 40 mL of PBS and filtering the rinsing liquid through the same filter membrane. Note: Initiate filtration as soon as possible (e.g., within 30 minutes). Two analysts are recommended to perform vortexing and filtration steps to reduce holding time after vortexing. c. Remove the membrane aseptically with sterile forceps and place it carefully over the surface of the recovery medium (trypticase soy agar for *P. aeruginosa* and *S. aureus*, Middlebrook 7H11 agar for *M. terrae*). Avoid trapping air bubbles between the filter and the agar surface. Incubate the plates for 24-48 hours at 36±1°C for *P. aeruginosa* and *S. aureus*, 21-28 days at 36±1°C for *M. terrae*. d. Examine the plates after incubation and record as CFU per carrier and – if necessary – per sample of the *Test Suspension A* or *B*. Calculate the averages for each set of test conditions with carriers.
13. Data Analysis/ Calculations	1. For the assay to be considered valid, ensure that: a. The recovered number of CFU in the **Titer Control with SL** (see

	section 12.4a) using *Final Test Suspension B* yields 20-200 CFU per carrier and, if necessary, when using the microbial suspension as a liquid. b. The recovered number of CFU in the optional **Titer Control without SL** (see section 12.5a) using *Test Suspension A* yields 20-200 CFU per carrier and, if necessary, when using the microbial suspension as a liquid. 2. For determining and verifying the effectiveness of the neutralizer, ensure that: c. The recovered number of CFU in the **Neutralizer Toxicity Control with SL** (see section 12.4b) is at least 75% of the **Titer Control with SL** (see section 12.4a). A count lower than 75% indicates that the neutralizer is harmful to the test organism. Note: counts higher than the **Titer Control with SL** (e.g., 120% of the **Titer Control with SL**) are also deemed valid. d. The recovered number of CFU in the **Neutralizer Effectiveness (with SL)** (see section 12.4c) treatment is within 75% of the **Titer Control with SL**; this verifies effective neutralization in the presence of SL in the inoculum. The same results are expected for the optional **Neutralizer Effectiveness (without SL)** (see section 12.5b) and **Neutralizer Effectiveness (independent addition of SL)** (see section 12.5c). Note: counts higher than the **Titer Control with SL** (e.g., 120% of the **Titer Control with SL**) are also deemed valid. 3. The criteria in sections 13.1 and 13.2 must be met. If the criteria are not met, another neutralizer or mixture of neutralizers must be identified and verified. 4. Always compare the numbers of the **Titer Controls** determined on carriers with the numbers of the respective **Neutralizer Toxicity Controls** or **Neutralizer Effectiveness** assays determined on carriers. If a liquid microbial suspension is used instead of carriers, compare the numbers determined in the respective suspensions with each other accordingly.
14. Forms and Data Sheets	1. Attachment 1: OECD Neutralization Assay Flow Chart 2. Test Sheets. Test sheets are stored separately from the SOP under the following file names: OECD Method for Bactericidal Activity: Neutralization Test Information Sheet — MB-26-00_F1.docx

	OECD Method for Bactericidal Activity: Neutralization Test Suspension Preparation Sheet — MB-26-00_F2.docx OECD Method for Bactericidal Activity: Neutralization Time Recording and Results Sheet (Required Components) — MB-26-00_F3.docx OECD Method for Bactericidal Activity: Neutralization Time Recording and Results Sheet (Optional Components) — MB-26-00_F4.docx
15. References	1. Draft Test Guideline: Quantitative Method for Evaluating Bactericidal Activity of Microbicides Used on Hard Non-Porous Surfaces (January 29, 2013).

Attachment 1

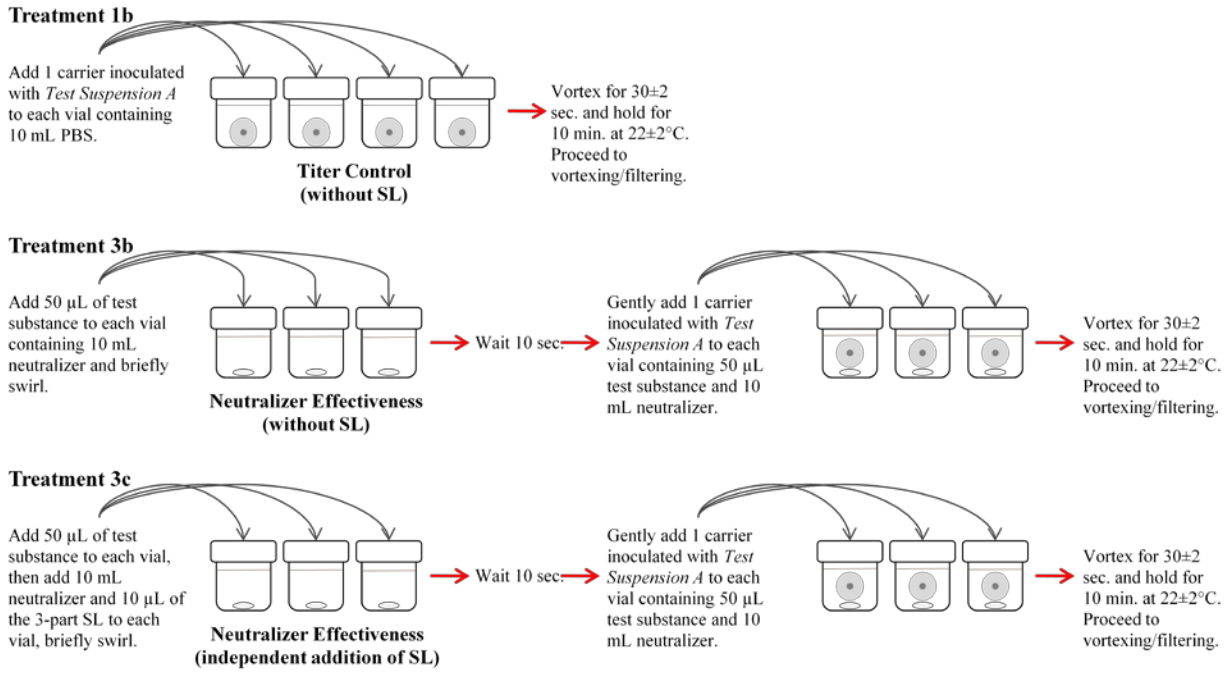
OECD Neutralization Assay Flow Chart (required components)



Optional suspension test: Using an additional vial, perform each procedure using 10 µL of the appropriate test suspension in place of the inoculated carrier.

Attachment 1 (cont.)

OECD Neutralization Assay Flow Chart (optional components)



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